Spring 2019

Ex Vivo Study of Skeletal Muscle Secretion of Interleukin-6

Anthony Drazick
University of South Dakota

Ex vivo study of skeletal muscle secretion of interleukin-6

By

Anthony T. Drazick

A thesis submitted in partial fulfillment
of the requirements for the
University Honors Program

Division of Basic Biomedical Sciences
Sanford School of Medicine
The University of South Dakota

April 2019

The members of the Honors Thesis Committee appointed
to examine the thesis of Anthony T. Drazick
find it satisfactory and recommend that it be accepted.

Dr. Yifan Li, M.D., Ph. D.
Associate Professor of Basic Biomedical Sciences
Director and Committee Chair

Dr. Kathleen Eyster, Ph. D.
Professor of Basic Biomedical Sciences
Committee member

Dr. Hong Zheng, M.D.
Assistant Professor of Basic Biomedical Sciences
Committee member

Abstract

Interleukin-6 (IL-6) was originally found as a cytokine in leukocytes that is involved in inflammation responses. With recent discovery of skeletal muscle endocrine function, several classical cytokines were found to be secreted from skeletal muscle, termed myokines. IL-6 has shown to be released from skeletal muscle as a myokine. As heterogenous tissue, skeletal muscle consists of at least four different types of muscle fibers characterized by distinct metabolic and functional properties. While IL-6 is known to regulate muscle metabolism and function, whether IL-6 release from skeletal muscle is muscle type specific is unclear. This study was designed (1) to validate an *ex vivo* method to test IL-6 release from isolated mouse skeletal muscle; and (2) to compare IL-6 release from slow and fast twitch muscle. Male and female C57/B6 mice at the ages of 3-4 months were anesthetized with isoflurane, extensor digitorum longus (EDL) (fast-twitch muscle) and soleus (slow-twitch muscle) were isolated and incubated in Krebs solution at 37 ºC with bubbling oxygen. Secreted IL-6 in the media was detected by the ELISA and western blot analyses. The results showed that IL-6 release was induced from isolated muscle in a temperature and O_2 dependent manner. IL-6 muscle release was cell-type specific with little secretion shown from the EDL but a strong release from soleus. Consistently, IL-6 protein secretion was greater in soleus tissue than that in EDL tissue. Moreover, while EDL expresses and releases little IL-6, it shows strong release of MMP-9 and other unidentified proteins. Overall, the results of this study suggest that skeletal muscle release of IL-6 is muscle fiber type specific.

Acknowledgments

I would first like to thank Dr. Li and everything he has done to help me be successful in writing my honor's thesis. I appreciate that Dr. Li let me volunteer in his lab in order to learn how research is done in a laboratory, how to do several different experiments, and how to properly use animals during research. I would also like to thank Ashley Liang and Hongbo Gao for helping me with experiments and showing me how improve on my research skills. Lastly, I want to thank my friends and family for supporting me during college and helping me stay motivated during such a busy time in my life.

Table of Contents

Table of Figures

1. Background

1.I. Skeletal muscle, a heterogenous organ, and its major fiber types

Skeletal muscle is the most abundant tissue in the body that performs with skeletal movement as a primary function; Skeletal muscle tissue is heterogenous or tissues with different characteristics depending on the body's needs **(Schiaffino, 2011).** Body's functional needs include a spectrum from continuous low-intensity movements (posture, long-distance running), to fast, maximal contraction movements (sprinting, jumping). While muscle composition is widely caused by phenotypic and genotypic factors, the nervous system can play a role in determining muscle fiber-type when muscle fibers form together to create a muscle (**Schiaffino, 2011).** In the human body, there are at least four types of muscle fibers, characterized by the distinct metabolism and function, type I, type IIa, type IIb, and type IIx. Studies show that different fiber-types have two different physiological types: speed of contraction and resistance to fatigue. Additionally, the different fiber-types show to have different enzymatic activity where type IIa and type IIb fibers have shown to have high levels of glycolytic enzymes (low levels of mitochondria, highly developed sarcoplasmic reticulum), and type-I fibers have shown evidence of fatty-acid oxidative enzymes which means high levels of mitochondria and lesser developed sarcoplasmic reticulum (**Schiaffino, 2011)**. Furthermore, studies have shown difference among type IIa and type IIb muscle fibers. Type IIa muscle fibers are fast-twitch, oxidative fibers that resist fatigue better than type-IIb fibers that are fast-twitch, glycolytic fibers that are quick to fatigue. These three fiber types are important when studying how the body moves with performance and efficiency. However, there is an additional type II, fast-twitch muscle fiber that was discovered known as type IIx which has similar glycolytic characteristics to type IIa and type IIb fibers but resists fatigue intermediate to them **(Schiafino, 2011)**. Muscle-type distribution throughout the

body varies among species, sex, and heredity and a mix of the fiber types is helpful for efficient but powerful movement depending on the body's necessities. When studying muscle physiology, it is important to look at different fiber-types to better understand the differences among them with regards to function and action.

1.II. Skeletal muscle secretory function

Recent studies show that muscles not only produce contractions that help with skeletal movement but also have secretory functionality to release myokines, cytokines that are secreted by muscle tissue **(Pedersen and Fischer, 2007)**. Evidence points to muscular communication with other tissues throughout the body by secreting myokines into the blood stream. According to Pedersen et. al., myokines communicate with other tissues (i.e. endocrine and paracrine) as well as communicate within the muscle tissue (autocrine) to signal hypertrophy, myogenesis, or AMPK-mediated fat-oxidation. Examples of myokines include IL-6, brain derived neurotropic factor (BDNF) and IL-7, and there are many more that have exclusive endocrine functionality in the muscle tissue. There are also myokines that help with circulation such as IGF-1 and FGF-2 which are just some examples. The overall effects of these myokines seem to be related to pancreas function and cancer growth **(Pedersen, et al. 2009)**. Exercise induced release of these myokine may have protective characteristics to prevent diseases that are correlated with a lack of exercise.

1.III. Interleukin-6 as a myokine

Interleukin-6 is an interleukin which is a cytokine that was originally found to be secreted by white-blood cells and helps to regulate cell differentiation, growth, and activation during an immune response (**Brocker, et al. 2010)**. Because of continuous evolution of white-blood cell DNA composition, it is difficult to classify interleukin cytokines; however, evidence suggests

that IL-6 assists with inflammation, cell-survivability, and cell-differentiation for leukocytes. Emerging evidence shows that IL-6 is also produced and secreted in a variety of non-immune tissues, including skeletal muscle. IL-6 myokine secretion is said to have anti-inflammatory function and its secretion is 100-fold during muscular exercise (**Febbraio and Pedersen, 2005)**. Surfacing data is showing that skeletal muscle is a major influencer in metabolism by myokine secretion **(Ling, et al. 2018)**. While IL-6 enhances insulin production during post-exercise, IL-6 can also be linked to obesity and reduced insulin action (**Febbraio and Pedersen, 2008).** Studies show that muscle-derived IL-6 secretion during exercise could be a signal that glycogen levels are "critically low" and that blood glucose is the main source of fuel for the active skeletal muscles.

Skeletal muscle tissue is a major component of the body by encompassing over 40% of total body mass. The muscle endocrine functions are being widely studied as these myokines help to regulate muscular performance, metabolism, and myogenesis pathways. Not only does IL-6 play a role in glucose uptake by muscle cells, it will also stimulate a lipolysis pathway by phosphorylating AMPK (AMP-kinase) in fat cells (**Febbraio and Pedersen, 2008).** Not only could a greater understanding of IL-6 help understand its functional mechanisms, but it could also help clinicians when helping patients with metabolic disorders. Given the important role of muscle derived IL-6 in regulation of muscle performance and metabolism, it is surprising how little is known regarding IL-6 secretion in different muscle fiber types.

In the present study, mouse soleus muscle, which primarily consists of slow oxidative (type I) fibers, and extender digitorum longus (EDL) muscle which mainly has fast glycolytic (type IIb) fibers were isolated, incubated, and stimulated to characterize IL-6 release.

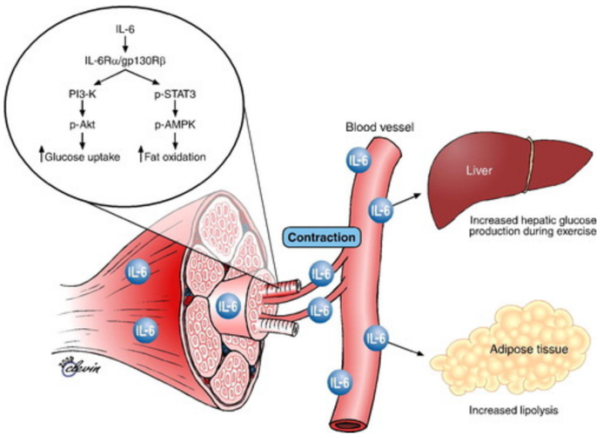

FIG. 4.

Fig.1 IL-6 release from skeletal muscle (**Pedersen and Fischer, 2007)**

Figure one represents a diagram of the effects that IL-6 has on systems of the body. After skeletal-muscular contraction, IL-6 regulates multiple pathways such as increased glucose uptake in the muscle cells, increased glucose production in the liver, and increased lipolysis or fat-oxidation in the adipose tissue. Not only will these pathways benefit the body's performance as a whole, the body could see other benefits such as a decrease in body weight due to oxidation of adipose tissue and increased glucose use in muscle tissue. IL-6 is important to study now that scientists have proven that muscle tissue secretes this myokine because of its potential use in medical treatment for patients with metabolic disorders, muscle diseases, or other possible problems **(Pedersen)**.

1.IV. Brain Derived Neurotrophic Factor released from skeletal muscles

BDNF is a growth factor in the nervous system that is associated with learning, memory, and neural plasticity **(Pedersen et al. 2009)**. Recent evidence has shown BDNF as an important regulator of metabolism in peripheral tissue. Furthermore, BDNF has been shown to be released from the skeletal muscle tissue and is now considered a myokine as well. According to Pedersen et. al., BDNF was shown to be a cofactor for stimulation of AMPK, and acetyl coenzyme A and has been shown to increase fatty acid oxidation *in vitro* and *in vivo*. BDNF is another myokine that is being studied in Dr. Li's laboratory. In the present study, BDNF release from the soleus and EDL were also tested in comparison with IL-6.

The overall hypothesis of this study was that IL-6 and BDNF release may be different from slow and fast muscle fibers.

2. Methods

2.I.: **Muscle isolation and ex vivo incubation**:

Adult male and female C57/ B6 mice were used at the ages of 3-4 months. The protocol for use of animals was reviewed and approved by USD IACUC and was in compliance with NIH guideline of use for animals in research. Isoflurane was used to anesthetize the mice, the skin of the hind limb was removed, and soleus and EDL were isolated, dissected from the body, and individually transferred to a Krebs solution containing bubbling oxygen.

2.II. Induction/inhibition of IL-6 release

Each sample of EDL or soleus muscle was incubated using 150 microliters of fresh Krebs solution in a tube. A stimulation cocktail (Thermo-Fisher Inc, # 00-4970-03), secretion transport inhibitor (# 00-4980-03), or the stimulation cocktail plus the inhibitor (# 00-4975-93) were added into the solution at 1:500 dilution. Equal volume of Krebs solution was added to the control sample. Each tube was incubated at 37º C with oxygen bubbling for one hour. One experiment tested the effect of temperature on IL-6 release, tubes were incubated at room temperature (~20º C) for one hour with oxygen supply. Another experiment tested of effect of oxygen on the release of IL-6 and tubes were incubated at 37º C for one hour without bubbling oxygen.

2.III. Detection of release IL-6 in incubation media and muscles (Western blot and ELISA)

After the one-hour incubation period, media and muscle tissue were collected. Protein levels of IL-6 or BDNF in the collected media were detected using ELISA kit (Biolegend, Inc.)

or Western blot using an antibody for IL-6 or BDNF (Santa Cruz Biotechnologies). The muscle tissues were homogenized in a RIPA buffer containing protease and phosphatase inhibitor cocktail (Thermo-Fisher, Inc.) After 10,000 X g centrifugation for 10 minutes, concentration of protein in the supernatant of homogenates was determined and then normalized. Proteins of interest, including IL-6 and BDNF, were determined using the standard western blot technique as described before using their respective antibodies **(Liang, et al. 2018)**.

2.IV. Immunofluorescence staining for IL-6 in muscle

In order to detect IL-6 in the muscle fibers, we used immunofluorescent staining. Fresh muscle tissues that were dissected were frozen on dry-ice. After, the tissues were embedded with optimal cutting temperature compound (OCT, Sakura Finetek USA, Inc.) and sectioned at 10 micrometer thickness using a cryostat (Leica). Next, the tissue was fixed in 4% formaldehyde for 20 minutes and washed multiple times with PBST. The tissue sections were then blocked with 1.5% BSA at room temperature for an hour and then incubated with primary antibody against IL-6 (rabbit antibody, ProteinTech, Inc.) at 4º C overnight. After more washes, the sections were incubated with a secondary antibody against rabbit Ig conjugated with fluorescent dye. The final slide images were taken using a fluorescent microscope (Olympus).

2.V Data analysis and statistics

ELISA quantification of IL-6 in the media was presented as mean ± standard deviation. The statistical comparisons were performed using one-way ANOVA followed by post hoc student's t-test, P-values less than 0.05 were considered statistically significant.

3. Results

3.I IL-6 was inducible from muscle in ex vivo condition

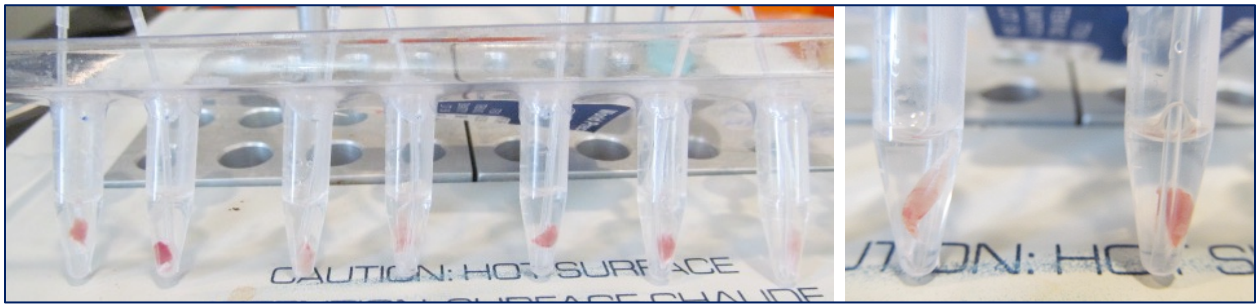

Fig. 2. *Ex vivo* incubation of isolated mouse soleus (darker) and EDL (lighter). Muscle tissues were isolated from mouse and placed into individual tubes with bubbling oxygen and Kreb's solution. The metal piece behind the tubes is the incubation chamber that will keep the muscle samples at 37 ºC for one hour.

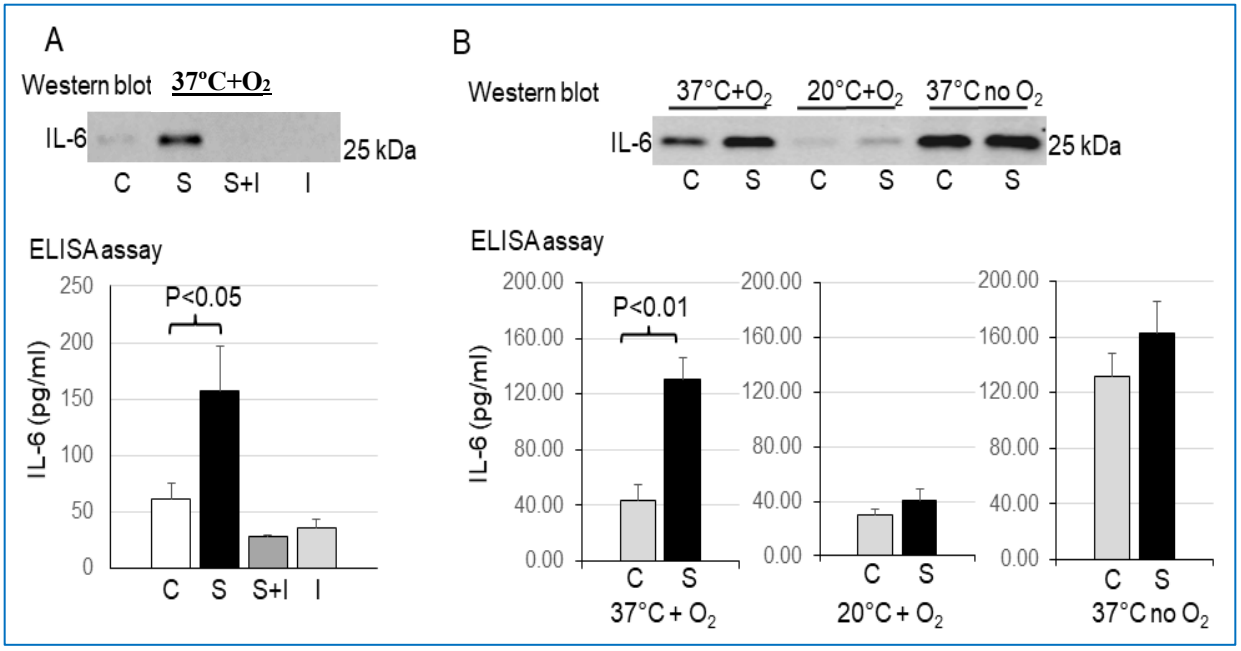

Fig. 3. IL-6 was inducible in *ex vivo* incubation. A: Western blot (top) and ELISA quantification (bottom) of IL-6 in the media of control (C), treatment with cytokine release stimulant cocktail (S), treatment with the stimulant and a cytokine release inhibitor (S+I), and the inhibitor alone (I), ELISA assay, n=4. B: Western blot (top) and ELISA quantification (bottom) of IL-6 in the media; control (C), stimulant (S) with 37 ºC and oxygen supply conditions (left), 20 ºC with oxygen supply conditions (middle), and 37 ºC without oxygen supply (right), ELISA assay, n=4.

Figures 2 and 3 demonstrate a validation of the *ex vivo* approach for the measurement of IL-6 release from skeletal muscle. The isolated muscles were incubated at 37 ºC with constant oxygen supply and were either stimulated, not stimulated, or treated with a stimulant inhibitor. As shown in panel A of figure 3, little IL-6 was secreted in control conditions, but a strong IL-6 release was induced by the stimulant, which was abolished by the secretion inhibitor. These results are consistent for the western blot and ELISA analyses. These results suggest that the IL-6 release was inducible and there was not spontaneous leak. The purpose of this experiment was to confirm that the *ex vivo* approach for IL-6 muscle secretion study was functional.

To further demonstrate that the *ex vivo* method was a physiological release, we tested different physiological conditions to compare release of IL-6. Detailed in panel B of figure 3, IL-6 release was studied with changes to temperature or changes to oxygen supply. Under normal physiological conditions (37 ºC + oxygen), the stimulated muscle tissue released a significant amount of IL-6. When the muscle was incubated in 20 ºC with oxygen, there was minimal release of IL-6 in the stimulated muscle, similar to the control muscle in these conditions. This shows the importance of physiological temperature for optimal release of IL-6. Additionally, when the muscle tissue was at physiological temperature but was incubated without oxygen, there was significant release in both control and stimulated muscle. These studies demonstrate the importance of physiological conditions for proper release of IL-6 and shows that an *ex vivo* study is a viable method to measure release of IL-6 in skeletal muscles.

3.II IL-6 was inducible from soleus but not EDL

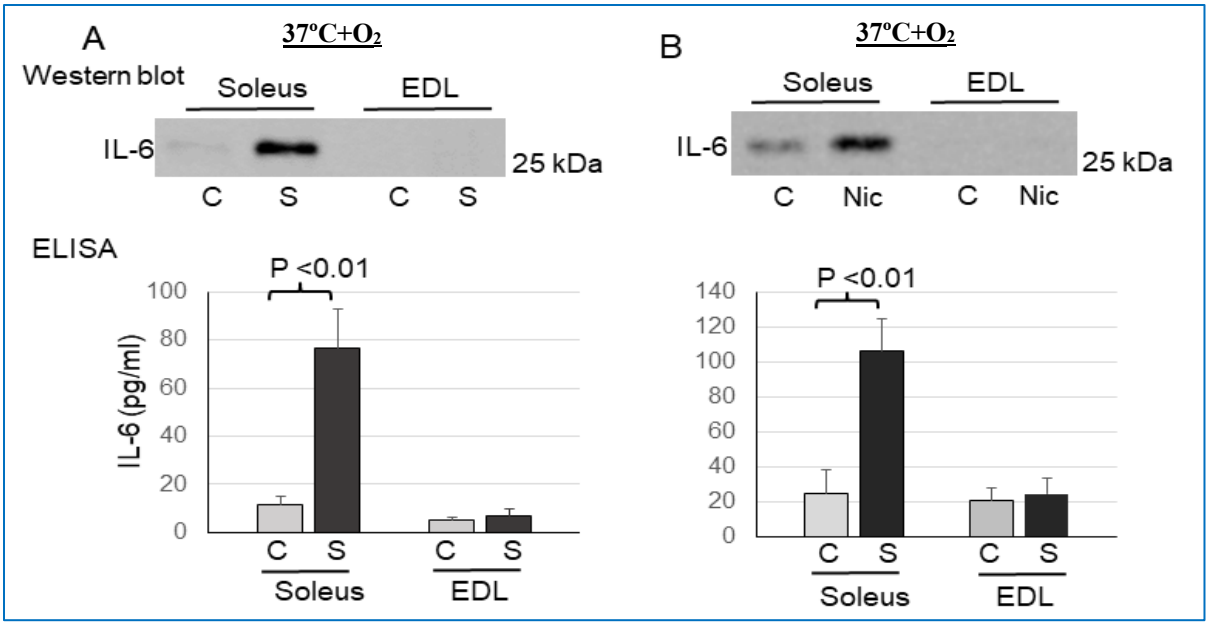

Fig 4. IL-6 was inducible from soleus but not from EDL muscle. A: Western blot (top) and ELISA quantification (bottom) of IL-6 release into the media from the soleus or EDL muscles, control (C) or treatment with cytokine release stimulant cocktail (S), ELISA assay, n=4. B: Western blot (top) and ELISA quantification (bottom) of IL-6 release into the media from the soleus or EDL muscles, control (C) or treatment with nicotine (Nic), ELISA assay, n=4. Nicotine was used to stimulate the nicotine-cholinergic receptor on the muscle to compare with the stimulation cocktail used in panel A.

Using the *ex vivo* method that we demonstrated to be viable in 4.I, we compared IL-6 release from the soleus, a slow twitch muscle, and EDL, a fast twitch muscle. Isolated muscle samples were incubated for one hour as a control or with the stimulation cocktail. While the stimulation cocktail was able to induce a substantial amount of IL-6 in the soleus, there was no induction of IL-6 release in the EDL. This finding suggests that IL-6 myokine release is muscle type specific and not arbitrary.

Furthermore, to test if the EDL and soleus muscles could be induced for IL-6 release by other stimulants, we stimulated the incubated muscles with nicotine (1 μM) which is an agonist for nicotinic cholinergic receptors on a neuromuscular junction. Similar to the stimulation

cocktail, nicotine was able to induce IL-6 release from the soleus but not from the EDL which adds further evidence for muscle type specific release of IL-6.

3.III IL-6 protein level was different in soleus and EDL tissues

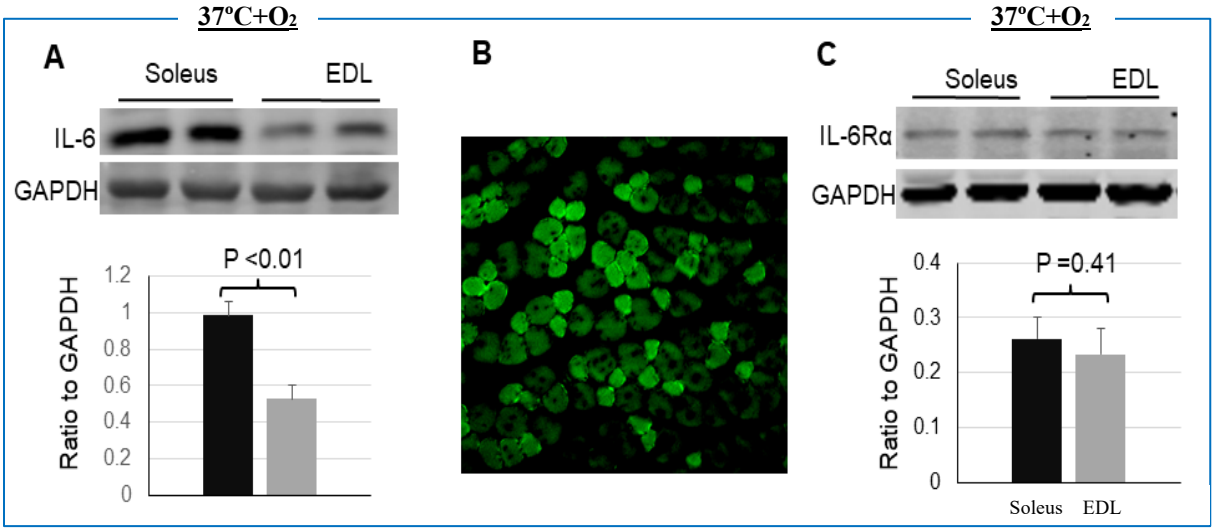

Fig 5. IL-6 protein level in muscle tissues. A: Western blot image (top) and quantification (bottom) of IL-6 and GAPDH protein levels in soleus (black bar) and EDL (grey bar). B: immunofluorescence staining of IL-6 in gastrocnemius muscle showing stronger signal in the oxidative fibers (smaller fibers). C: Western blot image (top) and quantification (bottom) of IL-6 receptor alpha and GAPDH in soleus (black bar) and EDL (grey bar).

Our next test was to see if IL-6 protein concentration within the muscle tissue is different between the two muscle types. Western blot analysis between the soleus and EDL muscle tissue was performed which showed significantly more IL-6 protein in the soleus tissue when compared to the EDL tissue. Also, immunofluorescent staining of the gastrocnemius muscle, which contains oxidative slow-twitch muscle fibers and glycolytic fast-twitch muscle fibers, , showed a good IL-6 signal in the slower oxidative fibers (smaller size) compared to the faster glycolytic fibers (larger size), which indicates IL-6 is expressed in greater amounts in the slower oxidative muscle fibers. Protein level of IL-6 receptors (IL-6Rα) was comparable between the two muscles. Future immunofluorescence staining will be done on EDL and soleus to see IL-6 signaling in different muscle types.

3.IV BDNF release was also inducible in soleus but not EDL

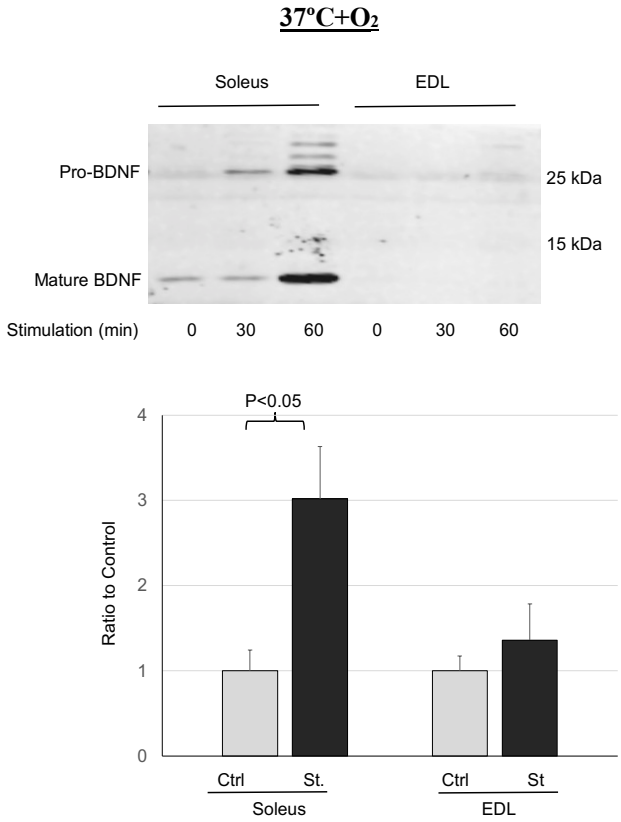

Fig 6. BDNF released from soleus and EDL. Western blot analysis (top) of muscle incubation media (incubated for one hour). Pro-BDNF and mature BDNF were induced to release from the soleus but EDL was not inducible. ELISA quantification (bottom) for BDNF in muscle incubation media with or without stimulation, ELISA assay, n=4.

BDNF showed similar results to the IL-6 myokine as they both were inducible from the soleus but not from the EDL. Using the same viable method as the IL-6 experiment, we stimulated the soleus and EDL under physiological conditions. The Western blot and ELISA analysis demonstrated that soleus secreted a significant amount of BDNF, but the stimulated EDL release a little BDNF, similar to the control.

3.V Soleus and EDL have different secretome (secretory function)

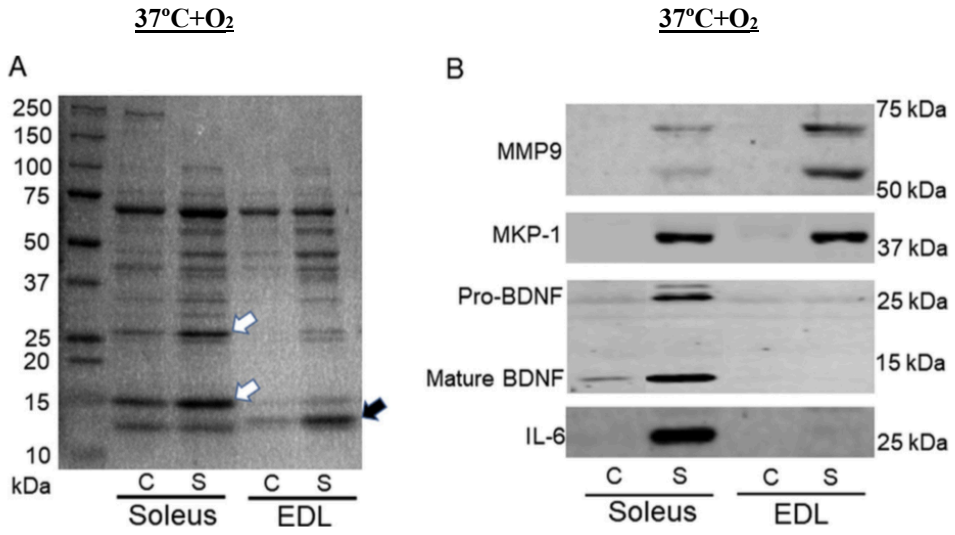

Fig. 7 Soleus and EDL have different secretome. A: gel Coomassie staining of proteins in the media of soleus and EDL with control or stimulated muscles. The white arrows indicate bands that are secreted from soleus and the black arrow indicates a band secreted from EDL. B: western blot images of MMP9, MKP-1, BDNF, and IL-6 in incubation media of soleus or EDL of control (C) or stimulation (S).

A question that was raised from the lack of IL-6 release and BDNF release in the EDL muscle was if EDL muscle had secretory function at all. We compared the total amount of proteins in the media of the soleus and EDL before and after stimulation. According to the gel Coomassie staining, the stimulation increased total secreted proteins in both the soleus and EDL muscles. Additionally, there were some protein bands that were different in the EDL compared to the soleus which suggests that EDL still has secretory function but with different secretome profile from that of soleus. This suggests that the secretome is different between the two muscles but that they both respond to the same stimulation. As an exmple, in western blot, IL-6 and

BDNF were only secreted from the soleus, not from EDL. However, MMP-9 was secreted with greater amount from the EDL than the soleus. MKP-1 was secreted similarly between the soleus and EDL. This data suggests that glycolytic and oxidative muscle fiber types have secretory function but the secretome is different between the two.

4. Discussion

IL-6 secretion from skeletal muscle was suggested to increase during exercise which was confirmed by study of primary muscle cells **(Pedersen and Fischer, 2007)**. While skeletal muscle is a heterogeneous tissue with different metabolic and contractile characteristics, it is unknown if IL-6 release is muscle type specific. With current *in vitro* and *in vivo* technologies, it is difficult to study IL-6 release. Our *ex vivo* model was effective when studying muscle secretion in different muscle types. A critical question that must be addressed is whether the myokine release from the isolated muscle is functional secretion or spontaneous leak. Our data show that the *ex vivo* IL-6 release was dependent on physiological conditions and blocked by an inhibitor, which gives evidence that the *ex vivo* IL-6 release was physiological and not spontaneous leak. Slow, oxidative tissues released IL-6 at 37 ºC with bubbling oxygen supply in Krebs solution and stimulation cocktail while fast, glycolytic tissues did not induce release of IL-6 under the same conditions. Under similar conditions but with 20 ºC incubation, IL-6 was not released from soleus or EDL which demonstrates more evidence for this effective model. Our effective model showed the necessity for appropriate physiological temperature, constant oxygen supply, and undamaged muscle tissue. Furthermore, this approach can be used to study different myokines under different types of stimulants like nicotine.

Using this effective model, we studied the difference in IL-6 release in the soleus and EDL muscles. EDL is a good example of a fast, glycolytic muscle, and soleus is a good example of a slow, oxidative muscle, and these two muscles were extracted from anesthetized mice of ages 3-4 months. These two muscles are good to study as they are easy to be isolated, and they are similar in size. Our evidence demonstrated that IL-6 was only inducible from the stimulated soleus muscle and not from the EDL. Furthermore, immunofluorescence of the gastrocnemius muscle, a mix of glycolytic and oxidative fibers, showed that IL-6 signal was higher in the smaller oxidative fibers as compared to the bigger glycolytic fibers that showed little to no signal for IL-6. We also tried comparing the normal cytokine stimulation cocktail with nicotine, a nicotine cholinergic stimulant for the neuromuscular junction, and these both showed similar results for the release of IL-6 from the soleus but not EDL. Still unknown to the experiment is if glycolytic fibers can release IL-6 under different metabolic conditions or stimuli. A possible test for this response would be to incubate glycolytic muscle with insulin in order to see the different response for glucose/glycolysis levels in the muscle which may help with release of IL-6 in the fast muscle. This knowledge would be beneficial for the study of IL-6 role in metabolism and how it is regulated in the skeletal muscle for different fiber types.

An important precursor to these experiments was to prove that glycolytic muscle fibers can still have myokine secretory function. We found that the total proteins released from the EDL was comparable to the soleus and both increased during stimulation. This demonstrates that EDL still has secretory function when stimulated, but that the response may be different than in the soleus. Furthermore, the Coomassie gel showed that EDL and soleus had different protein banding under stimulation which shows different responses to stimulation. After further study with different antibodies in the western blot analysis, soleus was shown to release BDNF and IL-

6 while EDL released MMP-9 more than soleus. MKP-1 release was similar between the two muscle types. Slow fibers and fast fibers have similar response to stimulation, but the proteins released are different. This phenomenon is potentially important as in the clinic there could be metabolic deficiencies linked with BDNF and IL-6 that could be better treatable with better understanding of these myokines. The *ex vivo* model will be helpful in further studying myokine release and its function within the skeletal muscle tissue.

In summary, this study validated an *ex vivo* method for study of myokine release from isolated muscle. The experiments using this method identified that IL-6 and BDNF were specifically released from slow oxidative muscle but not fast glycolytic muscle. This method and information are important for the future study of regulation and function of myokine secretion.

Funding

This study was supported by grants National Institutes of Health grant number 1R15HL118696, 1R03AG051926, and P20GM103443]; USD CBBRe pilot grant, and USD SSOM faculty research grant.

References

Brocker, Chad., Thompson, David., Matsumoto, Akiko., Nebert, Daniel W., Vasiliou, Vasilis. Evolutionary divergence and functions of the human interleukin (IL) gene family. *Human Genomics,* Vol. 5, No. 1, pp. 30-55, 2010.

Febbraio, M.A., and B.K. Pedersen. Contraction-induced myokine production and release: Is skeletal muscle an endocrine organ? *Exerc. Sport Sci. Rev.*, Vol. 33, No. 3, pp. 114–119, 2005.

Febbraio, M.A., and B.K. Pedersen. Muscle as an Endocrine Organ: Focus on Muscle-Derived Interleukin-6. *Physiological Reviews*, Vol. 88, No. 4, pp. 1379-1406, 2008.

Iizuka, Kenji., Machida, Takuji., Hirafuji, Masahiko. Skeletal Muscle Is an Endocrine Organ. *Journal of Pharmacological Sciences*, Vol. 125, pp. 125-131, 2014.

Liang, AP., Drazick, AT., Gao H., Li Y. Skeletal muscle secretion of IL-6 is muscle type specific: Ex vivo evidence. *Biochemical and Biophysical Research Communications,* Vol. 505, No. 1, pp. 146-150, 2018.

Pedersen, BK. Fischer, CP. Beneficial health effects of exercise--the role of IL-6 as a myokine. *Trends Pharmacol Sci.* Vol. 28, No. 4, pp. 152-156, 2007.

Pedersen, Brente K., Pedersen, Maria., Krabbe, Karen S., Bruunsgaard, Helle., Matthews, Vance B., Febbraio, Mark A. Role of exercise-induced brain-derived neurotrophic factor production in the regulation of energy homeostasis in mammals. *Exp. Physiol*, Vol. 94, No. 12, pp. 1153-1160, 2009.

Schiaffino, S. Reggiani, C. Fiber Types in Mammalian Skeletal Muscles. American Physiological Society, Vol. 91, No. 4, 2011.